Nucleic Acid Synthesis in the Chloroplasts of Acetabularia mediterranea\textsuperscript{1,2}

C. J. Chapman\textsuperscript{3}, N. A. Nugent\textsuperscript{4}, and R. W. Schreiber

Botany Department, University of New Hampshire, Durham

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Summary. Radioautographic and radiochemical techniques were used to establish the presence of replicating DNA in the chloroplasts of Acetabularia mediterranea. These techniques also demonstrated that these chloroplasts synthesize RNA. It was found that label from thymine was also incorporated into DNA and RNA in these chloroplasts. With the establishment of protein and nucleic acid synthesis in Acetabularia chloroplasts, it is clear that these chloroplasts carry out those metabolic processes which are most characteristic of autonomous cells.

To demonstrate the presence and/or synthesis of DNA in chloroplasts, it is of the utmost importance to be able to eliminate completely any possibility of nuclear DNA's interfering with the determination. Acetabularia is the ideal organism for these studies; it can easily be enucleated, its cells live for several months in this enucleated state, and its size and growth habit make it ideally suited for the various manipulations required in the experiments.

Primary emphasis in studying chloroplast DNA has been placed on establishing the presence of the DNA in chloroplast preparations (2, 5, 6, 7, 9, 11, 18, 20).

There are many characteristics of chloroplasts that would lead one to expect that this DNA is an active DNA that is replicating and synthesizing RNA (22). For example, it has been recognized for some time that chloroplasts are capable of growing and dividing independently of the growth and division of the cell. N. itellla chloroplasts demonstrate this clearly (10) and in Acetabularia, the chloroplasts grow and divide in enucleated cells.

The presence of replicating DNA in chloroplasts was investigated using thymidine-\textsuperscript{3}H incorporation in Acetabularia (3), Clivia (16), Bilbergia (16), Euglena (19), Spirogyra (23), and Nicotiana (24). With the exception of the Acetabularia experiments (3), the uptake occurred in cells with the nucleus still present. The findings in the Acetabularia experiments (3) are open to some question. They were used in a later paper from the same group (4) to support the idea that Acetabularia chloroplasts contain no DNA and again in a more recent paper (2) to prove that these chloroplasts do contain DNA.

While thymidine is readily incorporated into DNA and is usually used in tracer studies concerning DNA (1, 8, 15), the free base thymine is not well utilized (14, 15, 17). It has been noted that thymine is incorporated into a nucleic acid fraction in Acetabularia (3).

It was the purpose of this study to establish conclusively the synthesis of DNA and RNA by chloroplasts of Acetabularia mediterranea.

Materials and Methods

Experimental Organism. Acetabularia mediterranea (Lamouroux) cultures were grown in artificial sea water medium according to the methods of Keck (12) as modified by Schreiber et al. (21).

Preparation of Acetabularia Cells for Radioautography. Nucleated and enucleated cells of Acetabularia were placed in 10 ml of medium containing 25 \textmu c of either thymidine-methyl-\textsuperscript{3}H or thymine-methyl-\textsuperscript{3}H (6.0 c/mmole). After varying incubation times, the cytoplasm was transferred to microscope slides by squeezing the cytoplasm from the cells with tweezers and smearing the drop on the slide. The slides were washed with cold 12\% trichloroacetic acid for 10 minutes, then with distilled water for 5 minutes. Some slides were then treated with deoxyribonuclease (3 mg/ml, with 0.001 M MgSO\textsubscript{4} added) for 3 hours at room temperature and washed with trichloroacetic acid for 5 minutes. Other slides were treated with ribonuclease (3 mg/ml) for 3 hours at room temperature and washed with trichloroacetic acid for 5 minutes. All slides were dried in vacuum for several hours.

Radioautography. Radioautographs were made according to Kopriwa and Leblond (13) using Kodak's NTB2 liquid emulsion.

Preparation of Acetabularia Cells for Radiochem-

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\textsuperscript{3}Present address: Botany Department, University of Rhode Island, Kingston, Rhode Island. 02882.

\textsuperscript{4}Present address: Department of Microbiology, Dartmouth Medical School, Hanover, New Hampshire.
Radiochemical Analysis. Analysis was carried out as outlined in figure 1. Two-tenths of a ml aliquots were dried on planchets and counted with a Lionel 455 Geiger-Mueller counter with a 2.5% efficiency for $^{14}$C.

**FIG. 1.** Radiochemical analysis procedure.
Results

Results with Thymidine. Radioautography with thymidine-methyl-\(^{3}\)H showed that the chloroplasts were the primary site of cytoplasmic label uptake. Nearly all the chloroplasts were labeled. There was no measurable difference between incorporation into nucleated and enucleated cells, even after extended incubation times. While the nonchloroplast areas within the cytoplasmic smears tended to show a higher background graininess than areas of the slide not covered by the smears, it seemed to be a uniform background with no concentrations of grains except over the chloroplasts. Most of the trichloroacetic acid-insoluble label was removable following deoxyribonuclease treatment. A small amount was removable following ribonuclease treatment.

The results of the thymidine-2-\(^{14}\)C experiments are given in table I. Control experiments demonstrated that the label was actually solubilized by the deoxyribonuclease and ribonuclease.

Results with Thymine. Radioautography with thymine-methyl-\(^{3}\)H again showed the chloroplasts to be the primary site of label uptake in the cytoplasm. Most of the trichloroacetic acid-insoluble label was again removable after deoxyribonuclease treatment. The results with thymine-2-\(^{14}\)C are given in table II.

DNA. Thymidine Results. The radioautographic results showed that the thymidine was being incorporated into the chloroplasts of Acetabularia mediterranea. Enzyme treatments showed that much of the label was incorporated into DNA. Incorporation of thymidine into DNA is generally accepted to indicate replication of that DNA.

In order to determine more accurately the amount of label that was incorporated into DNA, experiments were carried out using \(^{13}\)C-labeled thymidine. This also allowed a more accurate determination of the effect of enucleation on the incorporation. It can be seen from table I that roughly two-thirds of the label was removable following deoxyribonuclease treatment. The effect of enucleation on incorporation was found to be very slight. Even when cells had been enucleated for over 3 months prior to incubation with label there was no real significant drop in the amount of incorporation. Since enucleated cells do not generally survive much more than 3 months, it would appear that chloroplasts continue to synthesize DNA, and presumably grow and divide right up to the time that the rest of the cell dies.

The important findings from these experiments were that the chloroplasts do synthesize DNA, and

### Table I. Radiochemical Analysis with Thymidine-2-\(^{14}\)C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Expt no. 1</th>
<th>Expt no. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells enucleated for 2 weeks prior to incubation with label</td>
<td>Cells enucleated over 3 months prior to incubation with label</td>
</tr>
<tr>
<td></td>
<td>Radioactivity (cpm)</td>
<td>% Acid insoluble Removed by treatment</td>
</tr>
<tr>
<td>Deoxyribonuclease</td>
<td>31,550</td>
<td>60.4</td>
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<tr>
<td>Ribonuclease</td>
<td>19,350</td>
<td>7.1</td>
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<tr>
<td>Formic acid (pellet)</td>
<td>1305</td>
<td>25.7</td>
</tr>
<tr>
<td>Deoxyribonuclease</td>
<td>Nucleated cells</td>
<td>75.0</td>
</tr>
<tr>
<td>Ribonuclease</td>
<td>5125</td>
<td>22.3</td>
</tr>
<tr>
<td>Formic acid (pellet)</td>
<td>650</td>
<td>2.7</td>
</tr>
</tbody>
</table>

### Table II. Radiochemical Analysis with Thymine-2-\(^{14}\)C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Expt no. 1</th>
<th>Expt no. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells enucleated for 1 week prior to incubation with label</td>
<td>Cells enucleated over 3 months prior to incubation with label</td>
</tr>
<tr>
<td></td>
<td>Radioactivity (cpm)</td>
<td>% Acid insoluble Removed by treatment</td>
</tr>
<tr>
<td>Deoxyribonuclease</td>
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<td>41.1</td>
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<tr>
<td>Ribonuclease</td>
<td>4625</td>
<td>23.1</td>
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<tr>
<td>Formic acid (pellet)</td>
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<td>35.8</td>
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<tr>
<td>Deoxyribonuclease</td>
<td>Nucleated cells</td>
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<tr>
<td>Ribonuclease</td>
<td>9500</td>
<td>32.0</td>
</tr>
<tr>
<td>Formic acid (pellet)</td>
<td>7680</td>
<td>25.8</td>
</tr>
</tbody>
</table>
that they continue to do so for long periods of time in the absence of the nucleus.

**DNA. Thymine Results.** Investigators generally agree that the free base thymine is not well utilized in DNA synthesis in either plants or animals. Brachet (4), however, noted that thymine was incorporated into a nucleic acid fraction in *Acetabularia.* The results obtained in the investigation reported here confirm that chloroplasts of *Acetabularia* incorporate label from thymine into DNA and RNA. The most striking difference between the results using the thymine-2-14C and thymine-2-14C was the considerably higher residual label in the pellet with the thymine. This would indicate that the thymine undergoes much more rapid and extensive metabolic changes and is incorporated into fractions other than the nucleic acids.

**RNA. Thymidine and Thymine Results.** With both the thymidine-2-14C and thymine-2-14C an average of about 25% of the acid insoluble label was removable following ribonuclease treatment. Thus there was a definite synthesis of RNA in the system. Since radioautography showed some reduction in the amount of label in the chloroplasts following ribonuclease treatment, it can be assumed that the synthesis takes place in the chloroplasts. Further evidence for the RNA synthesis in the chloroplast can be found in the amount of ribonuclease removable label in those cells that had been nucleated for more than 3 months. Certainly after this time any cytoplasmic (nonchloroplast) RNA synthesis would have ceased. Also, when studying protein synthesis, Nogent et al. (in preparation) found that chloroplasts which had been treated to remove adsorbed cytoplasmic ribosomes showed no appreciable drop from the level of protein synthesis in untreated chloroplasts. This indicates that these chloroplasts have their own protein synthesizing system.

**Acknowledgment**

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**Literature Cited**


